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Extreme Stabilization of a Thermolysin-like Protease by an Engineered Disulfide Bond*

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The thermal inactivation of broad specificity proteases such as thermolysin and subtilisin is initiated by partial unfolding processes that render the enzyme susceptible to autolysis. Previous studies have revealed that a surface-located region in the N-terminal domain of the thermolysin-like protease produced by *Bacillus stearothermophilus* is crucial for thermal stability. In this region a disulfide bridge between residues 8 and 60 was designed by molecular modelling, and the corresponding single and double cysteine mutants were constructed. The disulfide bridge was spontaneously formed *in vivo* and resulted in a drastic stabilization of the enzyme. This stabilization presents one of the very few examples of successful stabilization of a broad specificity protease by a designed disulfide bond. We propose that the success of the present stabilization strategy is the result of the localization and mutation of an area of the molecule involved in the partial unfolding processes that determine thermal stability.

Several members of the bacterial genus *Bacillus* are known to produce extracellular neutral proteases (1–6) that resemble thermolysin, the extremely stable protease from *Bacillus thermoproteolyticus*. These so-called thermolysin-like proteases (TLPs)¹ consist of 300–319 residues and share similar structural and functional characteristics. The three-dimensional structures of thermolysin (7, 8) and the TLP produced by *Bacillus cereus* (9, 10) have been solved by x-ray crystallography. On the basis of these structures, reasonably accurate models of other TLPs have been built (11). Naturally occurring TLPs exhibit large differences in thermal stability (11) and the structural features causing these differences have been the subject of several site-directed mutagenesis studies (11, 12).

At elevated temperatures TLPs as well as subtilisins are irreversibly inactivated as a result of autolysis (13–15). Because of the broad specificity of TLPs (16), conformational features rather than sequence characteristics determine the sites of autolytic attack (17), and it has been shown that the rate of thermal inactivation is determined by the rate of local

unfolding processes that render the protease susceptible to autolysis (11–13, 15, 18, 19). Previous studies on autolysis of broad specificity proteases (13, 15, 17, 20) together with observations concerning the structural changes during protein unfolding (21, 22) suggest that the local unfolding processes that lead to autolysis involve solvent-exposed regions (17, 19, 20). Accordingly, it has recently been shown that the difference in stability between TLP of *Bacillus stearothermophilus* (TLP-ste) and the more stable thermolysin is determined mainly by amino acid differences at the surface (12). Furthermore, it turned out that the important mutations were clustered in a limited part of the N-terminal domain (especially residues 56–69) of the protein, illustrating the localized nature of the stability-determining unfolding processes (11, 12). One mutant that stabilized TLP-ste rather strongly was T63F (23) (see Fig. 1A). Based on these observations we decided to try to stabilize TLP-ste by introducing a disulfide bond in this critical area (preferably close to position 63), the rationale being that, in principle, a disulfide bond can reduce local mobility and unfolding more than any other type of mutation.

Disulfide bonds can make considerable contributions to the stability of proteins (24–26), an effect mainly attributed to the decrease of conformational chain entropy of the denatured protein (26–29). Many attempts have been made to increase protein stability by introduction of novel disulfide bonds (24, 27, 30–39). Some studies turned out to be successful (35–37, 39), whereas others did not give the expected results (30, 31, 34, 38). Disappointing results have been mainly attributed to side effects of the individual Xaa → Cys mutations (31, 34, 35, 38) and/or to the introduction of strain resulting from suboptimal geometry of the disulfide bridge (30, 32).

In the case of industrially important broad specificity proteases such as subtilisin (31, 33, 34) and TLPs (38), most attempts to stabilize these enzymes by the introduction of disulfide bridges have been unsuccessful. Only for one engineered disulfide bridge in subtilisin E has a considerable increase in thermal stability been reported (36). However, this disulfide bridge was not designed *de novo* but was designed on the basis of a disulfide bridge encountered in a naturally occurring, more thermostable subtilisin variant.

In the present study we show how TLP-ste can be stabilized dramatically by introducing a *de novo* designed disulfide bridge. Furthermore, we provide an explanation for the lack of success in earlier attempts to stabilize broad specificity proteases by engineered disulfide bridges.

MATERIALS AND METHODS

Reagents—1,4-Dithio-DL-threitol (DTT) and *N*-(3-[2-furyl]acryloyl)-Gly-Leu amide were purchased from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany), and urea was from ICN Biomedicals GmbH

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¹ The abbreviations used are: TLP, thermolysin-like protease; TLP-ste, TLP of *B. stearothermophilus*; DTT, 1,4-dithio-DL-threitol; PAGE, polyacrylamide gel electrophoresis; MOPS, 3-(*N*-morpholino)propane-sulfonic acid.

(Eschwege, Germany). 5,5'-Dithiobis(2-nitrobenzoate) was from SERVA Feinbiochemica GmbH (Heidelberg, Germany). All other chemicals used were of the highest quality commercially available.

Design of Disulfide Mutants—A three-dimensional model of TLP-ste was built exploiting the sequence homology with thermolysin (7, 8) as described previously (11). Most model building and structural studies were done using the program WHAT IF (40). Residues are numbered throughout this paper according to the sequence of thermolysin (1).

Sites for insertion of disulfide bridges were selected using the program SS-BOND (41) as described previously (38). This program uses the backbone coordinates from the three-dimensional model to select residue pairs on the basis of the calculated C_{β} - C_{β} distances. Subsequently, S_{γ} positions with ideal or nearly ideal geometries were generated for the selected pairs. An energy minimization procedure was used to select acceptable conformations. Acceptable residue pairs that were located in the stability limiting region in the N-terminal domain were visually inspected using the thermolysin crystal structure and the TLP-ste model. To minimize the risk of modelling errors, the Xaa → Cys mutations were chosen only in regions where thermolysin and TLP-ste are highly similar. The most promising candidate appeared to be G8C/N60C. This cysteine pair could be modelled with close to ideal geometry and is located close to the aforementioned position 63. The two cysteines, as well as the individual single mutations, were introduced in a cysteine-free variant of TLP-ste (C288L-TLP-ste, called wild type throughout this study) whose stability is nearly identical to that of unmutated TLP-ste (42).

Plasmids and Strains—Plasmid pGE530⁴² contains the gene encoding the C288L variant of TLP-ste, which was used as wild type in this study. The protease-deficient strain *Bacillus subtilis* DB117⁴³ was used as host for this plasmid, and its variants were obtained by site-directed mutagenesis. Cells harboring these plasmids were grown at 37 °C in TY broth containing 5 µg/ml chloramphenicol as antibiotic. The *Escherichia coli* strains WK6mutS and WK6⁴⁴ as well as XL-1 Blue MRF' (Stratagene GmbH, Heidelberg, Germany) and the plasmids pMa (46) and pBluescript II SK(+) (Stratagene) were used in site-directed mutagenesis procedures.

Site-directed Mutagenesis—The plasmid pGE530 was used as template for the construction of the N60C single mutant by site-directed mutagenesis via polymerase chain reaction (megaprimer method (45)). Polymerase chain reaction fragments containing the mutation were cloned into pBluescript II SK(+) (Stratagene), and restriction mapping was used to screen for mutant clones. The double mutant G8C/N60C was constructed using the pMa/c system (44, 46) as described previously (42), by simultaneous annealing of two mutagenic primers. The sequence of mutated DNA fragments was verified by dideoxy sequencing (47) before using the fragments to construct pGE530 variants containing mutated forms of the TLP-ste gene. The G8C mutant was obtained by cutting and ligating appropriate fragments of the wild type gene and the gene encoding the double mutant.

Expression and Purification of Wild Type and Mutant Enzymes—Wild type and mutant proteins were produced and purified as described previously (43, 48) using affinity chromatography on Bacitracin-silica. The enzymes were stored in elution buffer containing 20 mM sodium acetate, pH 5.3, 5 mM CaCl₂, 2.5 M NaCl, 20% (v/v) isopropanol, and 0.03% (w/v) sodium azide.

An alternative purification scheme was applied to samples to be used for CD measurements. After separation of cells from the fermentation broth (10 min at 6000 rpm, BECKMAN J2-HC), the supernatant was concentrated by ultrafiltration (FILTRON ProVario-3-System) using an 8-kDa membrane (Nova series, FILTRON), and proteins were precipitated with ammonium sulfate (85% saturation). The precipitate was collected by centrifugation (20 min at 20000 rpm, BECKMAN L8-60 M ultracentrifuge), redissolved in 0.05 M Tris buffer, pH 7.5, 5 mM CaCl₂ and dialyzed twice against the same buffer. The concentrated enzyme was further purified by chromatography on a DEAE-Sephacel (Pharmacia Biotech Inc.) column equilibrated with 0.05 M Tris buffer, pH 7.5, 5 mM CaCl₂. The enzyme was recovered in the flow through, and active fractions were concentrated by ultrafiltration (DIAFLO membrane) and further purified using gel filtration chromatography (either on Sephadex G75, equilibrated with 0.05 M Tris buffer, pH 9.0, 5 mM CaCl₂, or on HiLoad Superdex75, equilibrated with 0.05 M Tris buffer, pH 7.5, 5 mM CaCl₂, both column materials being from Pharmacia Biotech Inc.).

Both purification procedures yielded electrophoretically homogeneous enzyme (SDS-PAGE (49)). The specific neutral protease inhibitor phosphoramidon (50) (final concentration, 1 mM) was added to samples for SDS-PAGE to prevent autolysis of the enzyme.

Characterization of Enzymes—Thermal stability was expressed as T_{50} being the temperature of incubation at which 50% of the initial

activity of a 0.1 µM solution of purified enzyme in 20 mM sodium acetate, pH 5.3, 5 mM CaCl₂, 0.5% (v/v) isopropanol, 62.5 mM NaCl, 0.03% (w/v) sodium azide, and 0.01% (v/v) Triton X-100 was retained after 30 min. Initial and residual enzyme activities were determined using a casein assay at 37 °C as described previously (51). The assay was calibrated using a standard wild type enzyme preparation, and an arbitrary unit for protease activity was defined as the amount of activity required to increase the absorbance at 275 nm by 1 per minute under the conditions of the assay (52). The stability of mutant enzymes is given as ΔT_{50} , representing the difference in T_{50} between the wild type and mutant enzymes. The T_{50} values reported in this article differ slightly from previously published values as a result of some modifications of the assay conditions as used in Ref. 12. 0.01% (v/v) Triton X-100 was included in the assay mixtures to prevent unspecific binding of the protease to the surface of the reaction vessels.

The time course of thermal inactivation was followed using the same conditions as described above. The enzymes were incubated at defined temperatures, and the aliquots removed after different time intervals were assayed for activity toward casein at 37 °C.

The kinetic parameter k_{cat}/K_m for *N*-(3-[2-furyl]acryloyl)-Gly-Leu amide was determined according to the method of Feder (53) using a buffer containing 10 mM MOPS, pH 7.0, 5 mM CaCl₂, 0.02% (v/v) Triton X-100, 1% (v/v) isopropanol, and 125 mM NaCl. Activities were derived from the decrease in absorption at 345 nm, using a $\Delta\epsilon$ of 317 M⁻¹ cm⁻¹. The decrease in absorption was recorded at 37 °C, using a thermostatted cuvette in a Perkin-Elmer Lambda 11 spectrophotometer (Perkin-Elmer Corp.).

CD spectra were recorded using a JASCO J-710 circular dichroism spectrometer. Measurements were performed at 25 °C using a quartz cell of 1-mm path length. Samples contained 0.08 mg of enzyme/ml in 0.05 M Tris buffer, pH 9.0, 5 mM CaCl₂. Protein concentrations were determined with the Micro BCA protein assay reagent (Pierce) using bovine serum albumin as a standard.

Free thiols were determined according to Ellman (54) under denaturing conditions (6 M urea); the presence of free thiol groups in the G8C/N60C mutant was determined without or with previous incubation with reducing agents (0.2 M DTT). Excess of reducing agent was removed via extensive dialysis. The amount of free sulfhydryl groups was calculated using an extinction coefficient of 13,600 M⁻¹ cm⁻¹.

SDS-PAGE analysis of purified TLP-ste variants was performed using a method essentially similar to the method described by Laemmli (49). The presence of disulfide bonds was analyzed by comparing mobilities during SDS-PAGE of enzyme samples that had been prepared in the absence or the presence of reducing agent.

RESULTS

Design and Production of the Mutants—Fig. 1 (A and B) shows the 8–60 disulfide mutant as designed in the three-dimensional model of TLP-ste. The disulfide bond connects the N-terminal β -hairpin (residues 1–25) with a region that is crucial for thermal stability (residues 56–69) (11, 12). Inspection of the thermolysin crystal structure and the TLP-ste model indicated that the individual mutations needed for the disulfide bond (G8C and N60C) would not lead to significant clashes or have other negative side effects.

The selected mutants were constructed and could successfully be expressed in *B. subtilis* DB117. Wild type TLP-ste, G8C, and G8C/N60C mutants were similar with respect to expression levels and yields of purification. The expression level was approximately three times lower for the N60C mutant. Wild type and mutant proteins had similar specific activities toward casein as substrate at 37 °C, pH 7.5 (81.5 ± 5.3 units/mg, 82.3 ± 5.0 units/mg, 75.0 ± 5.2 units/mg, 82.4 ± 6.2 units/mg protein for purified wild type, G8C, N60C, and double mutant enzymes, respectively). The k_{cat}/K_m of the double mutant enzyme for the synthetic dipeptide substrate *N*-(3-[2-furyl]acryloyl)-Gly-Leu amide (53) was similar to that of the wild type enzyme (27.8 ± 3.5 · 10³ and 30.0 ± 4.4 · 10³ M⁻¹ s⁻¹, respectively).

Nonreducing SDS-PAGE showed that the double mutant enzyme migrated slightly faster than the wild type enzyme, whereas identical mobilities were observed in the presence of reducing agent (DTT; results not shown). This suggests that

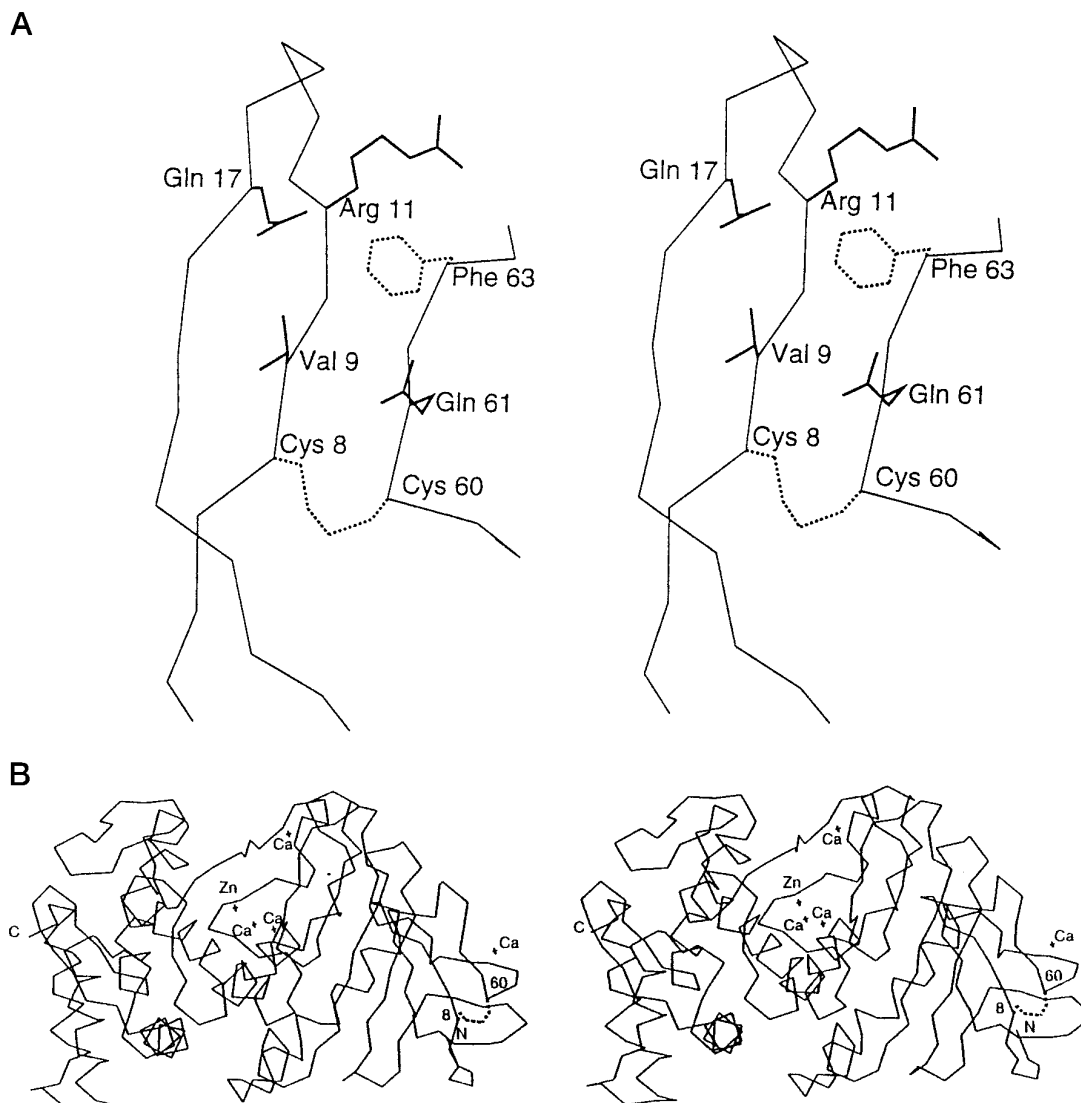


FIG. 1. **The engineered disulfide bond in TLP-ste.** A, detail of the TLP-ste model containing mutations G8C, N60C, and T63F (side chains as dotted lines). The side chains shown in bold lines interact with the side chain of residue 63, as described previously (23). B, C- α trace of the G8C/N60C double mutant of TLP-ste. Crosses indicate bound calcium and zinc ions (the zinc ion is located at the bottom of the active site cleft). The N and C termini are indicated.

the expected disulfide bond was formed *in vivo* in the G8C/N60C mutant. No free thiol groups could be detected by thiol titrations (under denaturing conditions) with Ellman's reagent (54), confirming the spontaneous formation of the disulfide bridge in the double mutant. After treatment with excess of DTT (0.2 M), the number of sulfhydryl groups in the double mutant was determined to be 1.95 ± 0.15 /molecule.

The CD spectrum of the double mutant was identical to that of the wild type enzyme (not shown), indicating that the tertiary structure had not changed significantly as a result of the introduced disulfide bond.

Thermal Stability—Purified, electrophoretically homogeneous wild type and mutant enzymes were used for determining T_{50} as described under "Materials and Methods." As shown in Table I, the single mutant enzymes were considerably less stable than the wild type enzyme (ΔT_{50} is -11.0 and -16.2 °C, for G8C and N60C, respectively). Reducing agents had a stabilizing effect on the single mutant enzymes but only a small effect on the wild type enzyme. This suggests that the decrease in thermal stability of the single mutants is at least partly due to oxidation of the introduced cysteine residue and, possibly, formation of intermolecular disulfide bonds (55).

Despite the destabilizations observed for the single mutants, the double mutant displayed a drastic increase in T_{50} ($\Delta T_{50} = +16.7$ °C). DTT reduced the stability of this mutant, but even at 10 mM DTT the mutant was much more stable than the wild type ($\Delta T_{50} = +11.8$ °C). Thus, it seems that the engineered disulfide bridge is rather resistant toward reduction. At higher DTT concentrations (50–100 mM) the stability of the double mutant was further reduced, but stability measurements at such high concentrations could not be performed accurately, because increasing DTT concentrations resulted in considerable decrease of the enzymatic activity in wild type and in all mutant enzymes.

In the temperature range of 80–95 °C for the stable double mutant enzyme and 55–75 °C for the unstable single mutant enzymes, the kinetics of thermal inactivation was measured and compared with those of the wild type TLP-ste. In all cases the inactivation was irreversible and followed a first order kinetics. Thermal inactivation of the double mutant coincided in the usual manner with the disappearance of protein material visible in SDS-PAGE gels (Fig. 2). The results (Table II) confirmed the low stability of the single mutants and the extreme stabilization obtained by introduction of the disulfide

TABLE I
Thermal stabilities of TLP-ste mutants

ΔT_{50} values represent the difference between the wild type and the mutant enzymes with respect to the temperature at which, after 30 min of incubation, 50% of the initial activity remains. The concentration of DTT used was 10 mM. The T_{50} values are the averages of at least three independent assays. Standard deviations were below 0.5 °C in all cases.

Mutant	T_{50}		ΔT_{50}	
	–DTT	+DTT	–DTT	+DTT
	°C			
Wild type	75.4	76.3	0	+0.9
G8C	64.4	70.7	–11.0	–4.7
N60C	59.2	67.5	–16.2	–7.9
G8C/N60C	92.1	87.2	+16.7	+11.8

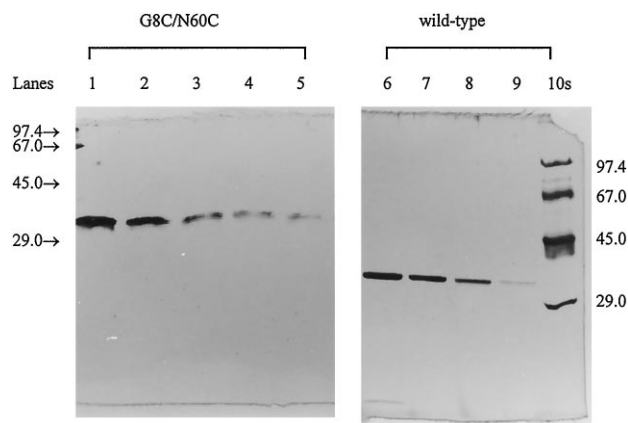


FIG. 2. Autolysis of TLP-ste variants at elevated temperatures. Gels were run under standard (reducing) conditions and stained with silver. Identical aliquots of proteins were incubated at high temperatures for different periods of time and subsequently subjected to SDS-PAGE. Lanes 1–5, the G8C/N60C mutant enzyme after 0, 10, 20, 30, and 40 min at 92.5 °C, respectively; lanes 6–9, the wild type enzyme after 0, 15, 20, and 30 min at 75 °C, respectively; lane 10, molecular mass marker (SERVA). Molecular masses of marker proteins are given in kDa; in the left panel the approximate positions of marker proteins are indicated by arrows.

bond. The half-life of the enzyme at 92.5 °C was increased more than 120-fold, from less than 0.3 to 36 min. Remarkably, even the stability of the most stable naturally occurring TLP, thermolysin, was exceeded considerably (Table II).

DISCUSSION

In the present study we provide the first example of drastic stabilization of a broad specificity protease by a *de novo* designed engineered disulfide bridge. In terms of kinetic stability, the disulfide containing mutant of TLP-ste is one of the most stable enzymes ever obtained by protein engineering. The stability of this designed mutant is comparable with the stability of a recently published mutant of TLP-ste in which five amino acids (all in the 1–70 region) had been replaced (12) by the corresponding residues in a naturally occurring more stable TLP variant (thermolysin).

Broad specificity proteases, in particular subtilisin, were among the first enzymes the stability of which was manipulated using protein engineering (31, 33, 34, 36, 38). Attempts to introduce disulfide bridges were successful in the sense that it turned out that, indeed, completely rational design of spontaneously forming disulfide bridges was possible (31, 33, 35, 36). However, the stabilizing effects of these mutations were disappointing (30, 31, 34, 38). Taking into account that the rate of thermal inactivation of broad specificity proteases is determined by the rate of local unfolding processes preceding autolysis (11–13, 18, 19) we propose that the lack of success of these engineered disulfide bridges is at least partly due to the fact

TABLE II
Half-lives of TLP-ste variants at 60 and 92.5 °C

Half-lives were determined by assaying residual activities at 37 °C after exposure of the enzymes to the indicated temperatures as described under “Materials and Methods.” The values are the averages of at least three independent assays; standard deviations are given. The half-life of the wild type enzyme at 92.5 °C could not be measured; the value given is an estimate from an Arrhenius plot based on data obtained in the temperature range of 60–80 °C.

Mutant	Half-life	
	at 60 °C	at 92.5 °C
	min	
Wild type	813 ± 31	<0.3
G8C	170 ± 7	
N60C	35.2 ± 1.9	
G8C/N60C		35.9 ± 1.5
G8C/N60C + 10 mM DTT		15.8 ± 0.8
Thermolysin		9.8 ± 0.5

that these bridges were introduced in regions of the protease molecule that do not play a role in stability-determining local unfolding processes. Designed disulfide bridges have been extremely successful in cases where the stability measurement was based on monitoring global (as opposed to local) unfolding (24, 35). In these cases, the success of the bridge is more exclusively determined by the success of the design, and much less (if at all) by the location of the bridge in the molecule. Unfortunately, the phenomenon of autolysis prevents analysis of reversible unfolding in TLPs (*e.g.* Ref. 13). In cases where stability is determined by autolysis or other irreversible mechanisms of inactivation that do not depend on complete unfolding, mutational effects are at least partly determined by the location of the mutation. Accordingly, the present study shows that unspecific proteases can be stabilized dramatically by introduction of disulfide bridges, provided that thorough stability studies have indicated where the bridge has to be introduced. Only bridges introduced in regions involved in the stability determining local unfolding processes will lead to stabilization, and if such a region is found, the resulting stabilization may be enormous.

It may be possible to stabilize TLP-ste even more effectively if the rate-limiting cleavage sites are known. However, as a result of the rapid degradation after the first cleavage, it has so far not been possible to isolate kinetically relevant autolytic breakdown products of TLP-ste.² The present and previous data (12) on engineering the stability of TLP-ste clearly show that the stability of this enzyme is determined by a part of the N-terminal domain (important mutations have been found between positions 1 and 70). It remains to be elucidated where the stability determining autolytic cleavages take place and what extent of local unfolding is needed for those to occur. The present study shows, however, that highly successful rational design of stabilizing mutations in TLP-ste is possible on the basis of the information that is currently available.

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